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Proteases and Protease Inhibitors Produced in Streptomycetes and Their Roles in Morphological Differentiation

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Streptomycetes are Gram-positive microorganisms producing secondary metabolites through unique physiological differentiation [4]. The microbes show unusual morphological differentiation to form substrate mycelia, aerial mycelia, and arthrospores on solid medium [19]. Substrate mycelium growth is sustaining with sufficient nutrients in the culture medium. The concentration of a specific individual substrate in the culture environment is the most important extracellular factor allowing vegetative mycelia growth, where extracellular hydrolytic enzymes participate in the utilization of water-insoluble substrates.

However, with starvation of nutrients in the culture medium, the vegetative mycelia differentiate to aerial mycelia and spores. It has been considered that shift-down of essential nutrients for mycelia growth is the most important factor triggering morphological and physiological differentiation in *Streptomyces* spp. Since proteineous macromolecule compounds are the major cellular components, these are faced to endogenously metabolize following a severe depletion of nitrogen source in culture nutrients (Fig. 1). Various proteases were identified of which production was specifically related with the phase of mycelium growth and also morphological differentiation. The involvement of proteases and protease inhibitor is reviewed as a factor explaining the mycelium differentiation in *Streptomyces* spp.

Proteases and Protease Inhibitors Produced in *Streptomyces* spp.

It was reported that *Streptomyces* can produce various kinds of extracellular proteases and also protease inhibitors. The proteases produced abundantly in *Streptomyces* spp. are classified according to active sites or a susceptible

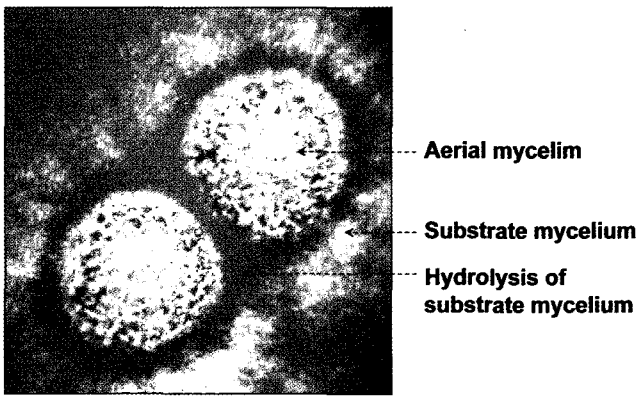

Fig. 1. Reuse of substrate mycelium by hydrolytic enzymes for morphological differentiation in *Streptomyces* spp.

Key words: *Streptomyces*, morphological differentiation, protease, protease inhibitor, leupeptin inactivating enzyme, chymotrypsin, metalloprotease, *Streptomyces griseus* metalloprotease A

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Abbreviations: **ANNs**, artificial neural network; **AdpA**, A-factor-dependent protein; **CTP**, chymotrypsin-like protease; **ELP**, elastase-like protease; **LIE**, leupeptin-inactivating enzyme; **MTP**, metalloprotease; **PI**, protease inhibitor; **PyMs**, pyrolysis mass; **SGAP**, *Streptomyce griseus* aminopeptidase; **SgmA**, *Streptomyces griseus* metalloprotease A; **SSI**, *Streptomyces* subtilisin inhibitor; **STI**, *Streptomyces* trypsin inhibitor; **TLCK**, tosyl lysine chloromethyl ketone; **TLP**, trypsin-like protease; μ, specific rate of mycelium growth; q_{PIs}, specific rate of PIs production; q_s, specific glucose uptake rate; q_{LIE}, specific rate of LIE production; q_{CTP}, specific rate of CTP production; q_{spo}, specific rate of spore formation; q_{TLP}, specific rate of TLP production

region of their substrates as follows: serine proteases, metalloproteases, and aminopeptidases. In the *S. coelicolor* genome, 27 serine proteases, 8 metalloprotease, and 21 aminopeptidases have been annotated [10].

Figure 2 represents a phylogenetic analysis of *Streptomyces* proteases including annotated *S. coelicolor* proteases. Serine protease family and metalloprotease/aminopeptidase family clusters were classified in the phylogenetic tree.

Serine Protease. Serine proteases can be divided into four groups according to substrate specificity: trypsin-like proteases (TLPs) that cleave after positively charged residues, subtilisin and chymotrypsin-like proteases (CTPs) that cleave after large apolar residues, elastase-like proteases (ELPs) that cleave after small nonpolar residues, and amino acid specific proteases that cleave after negatively charged residues like glutamic acid.

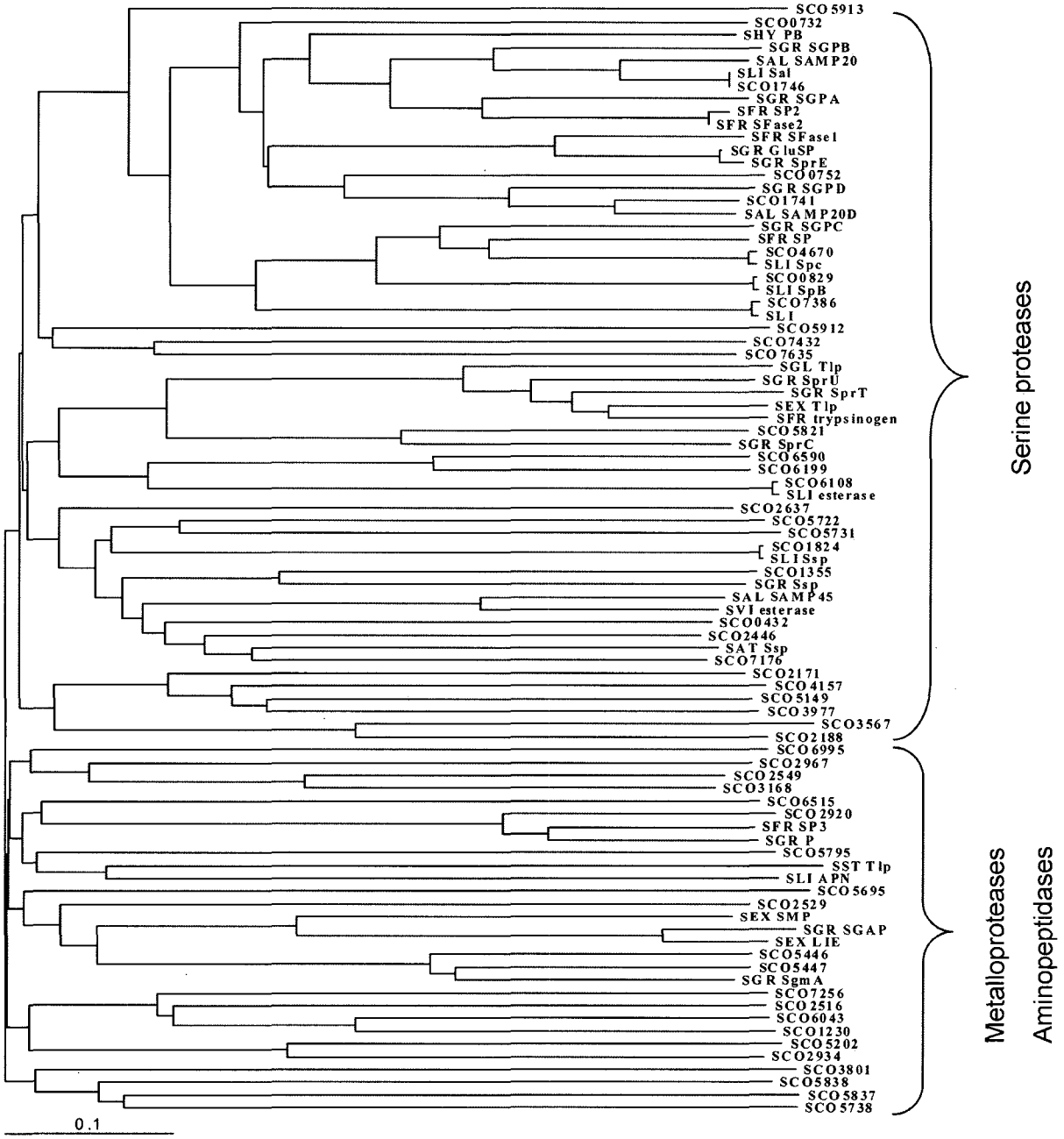

Fig. 2. Phylogenetic analysis of proteases produced in *Streptomyces* spp.
SCO number was taken from the *S. coelicolor* database annotation of the *S. coelicolor* genome sequence at http://streptomyces.org.uk. Abbreviations of strain name: SHY, *S. hygroscopicus*; SGR, *S. griseus*; SAL, *S. albogriseolus*; SLI, *S. lividnas*; SFR, *S. fradiae*; SGL, *S. glaucescens*; SEX, *S. exfoliatus*; SVI, *S. viridosporus*; SAT, *S. atroolivaceus*; SST, *S. steffisburgensis*.

TLPs are produced abundantly from *Streptomyces* spp. including *S. griseus* and *S. fradie* [25, 35, 68, 69]. TLPs produced from *S. griseus* have been well characterized with regards the enzymatic properties, molecular cloning of the gene, expression regulation, elucidation of the molecular structure, and modification of the structure, etc. [3, 47, 70–72], as well as trypsin of *S. erythraeus* [65, 96, 97].

CTPs are also widely produced in various species of *Streptomyces*. The major function of CTPs is to hydrolyze insoluble extracellular proteins as the nitrogen source for mycelia growth [30]. CTPs produced in *S. griseus* were characterized in terms of substrate specificity and molecular structure [6–8, 78]. The characteristics of subtilisin as a type of serine protease were identified from *S. spheroides*, *S. lividans*, and *S. albogriseolus* [16, 54, 80]. Another type of serine protease hydrolyzing after specific amino acid residues such as glutamic acid was also isolated from *S. griseus* and *S. fradie* [5, 49, 50, 77, 79, 81, 82].

Metalloprotease. Metalloprotease can bind metal ions such as Ca^{2+} or Zn^{2+} at the active site of the proteases. It has been found that the metal ions coordinate two or four side chains rendering the enzyme to have protease activity. Metalloproteases were identified from diverse species of *Streptomyces*. For instance, *S. lividans* produced five different types of metalloproteases (SlpA, SlpB, SlpC, SlpD, and SlpE), among which SlpD and SlpE were found to play essential roles on mycelia growth and cell viability [11, 13]. SlpA was also identified to be a novel class of neutral protease, of which expression was regulated by a transcriptional regulatory protein of a LysR family [13, 61]. Interestingly, *S. cacaoi* produced an extracellular zinc-containing neutral metalloprotease that was initially accumulated as a premature protein and then the premature protein was modified to form a mature protein *via* an autocatalytic cleavage mode [17, 18]. Additionally, two zinc-containing metalloproteases were identified from *S. griseus* [92], and a neutral metalloprotease was identified from *S. fradiae* [12]. A novel subfamily of zinc-containing metalloendoprotease was identified from *S. caespitosus* and the protease was found to be the smallest protease (132 aa) among those identified from *Streptomyces* spp. [24, 58].

Aminopeptidase. Aminopeptidase is an exopeptidase that is susceptible to N-termini of substrate proteins. The most well-characterized aminopeptidase is produced by *S. griseus* (SGAP), which is a zinc metalloenzyme activated by calcium ions [1, 9, 23]. A novel aminopeptidase found from *S. septatus* was compared with those obtained from *S. griseus* [2], and an aminopeptidase and tripeptydyl aminopeptidase were identified from *S. lividans* [14, 15, 55]. Furthermore, substrate specificity of the tri/tetra peptidyl aminopeptidase produced by *S. mobaraensis* was determined [94] and the tripeptidyl aminopeptidase was found to participate in the translational modification of transglutaminase [98].

***Streptomyces* Subtilisin Inhibitor.** It has been reported that *Streptomyces* spp. produce various types of protease inhibitors having either high or low molecular weight [63]. Among proteineous protease inhibitors, *Streptomyces* subtilisin inhibitor (SSI) was the first isolated from *Streptomyces* [63, 64, 75] and thereafter the SSIs were also isolated from other species of *Streptomyces* [27, 93, 95], and the structure of SSI was proposed [89, 90]. The SSIs are quite conserved in *Streptomyces* spp. [57, 83, 87] and show high similarity in amino acid alignment with about 100 amino acids.

It was found that SSIs inhibited various types of serine proteases such as subtilisin, chymotrypsin, and trypsin. In fact, serine proteases (chymotrypsin family) interacting with SSIs were identified in *S. albogriseolus* [80, 84–86, 88]. Furthermore, two zinc-containing metalloproteases (SGMPI and SGMPII) identified from *S. griseus* [92] were found to be strongly inhibited by SSI [53, 56]. The proteases inhibited by SSIs showed an identical catalytic triad. The inhibition specificity of the SSIs was determined by P1 and P4 sites of the reaction center [51, 52]. The SSIs isolated from various microorganisms are dimeric proteins. The inhibitors consist of two identical subunits that inhibit serine proteases by forming a tightly bound inhibitors-protease complex.

Extracellular Proteases and Protease Inhibitors Play Cascade Roles in the Morphological Differentiation of *Streptomyces exfoliatus* SMF13

A strain (SMF13) of *Streptomyces exfoliatus* was found to produce extracellular proteases and protease inhibitors (PIs) [38, 39]. The proteases obtained from the culture supernatant of *S. exfoliatus* SMF13 was chymotrypsin-like protease (CTP), metalloprotease (MTP), and trypsin-like protease (TLP). The production of proteases was very closely linked to the culture condition and medium formulation. Nitrogen source was the most critical ingredient affecting the production of proteases. The protease inhibitors (PIs) produced in the strain were found to be a mixture of N-acetyl-leu-leu-arginal, N-acetyl-ile-leu-arginal, and N-acetyl-leu-ile-arginal. The latter two molecules are new analogues of leupeptin [39].

CTP and PIs were produced in association with substrate mycelia growth, whereas TLP were progressively produced with an apparent decrease in the mycelium growth in submerged culture. PIs produced during the mycelium growth were inactivated when mycelia growth reached the stationary phase in submerged cultures. The TLP activity increased when PIs were completely inactivated by a new protein, leupeptin-inactivating enzyme (LIE), and autolysis of mycelia in the late stationary phase was apparently

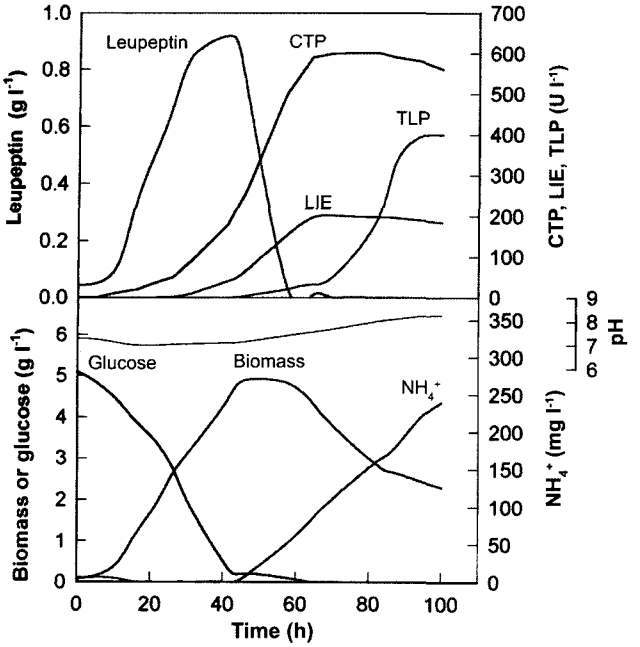

Fig. 3. Growth and production of proteases associated with cellular differentiation in *Streptomyces exfoliatus* SMF13.

linked with the increase of TLP activity [40]. The activity of LIE and TLP was high in the region of aerial mycelia or spores, but that of PIs and CTP was higher at the peripheral growing region [41] (Fig. 3). The quantitative relation between the medium composition and morphological differentiation was determined with the data obtained from batch and chemostat cultures. The specific rate of PIs production (q_{PIs}) was closely related to the specific glucose uptake rate (q_s) and specific rate of mycelium growth (μ). However, the specific rate of LIE production (q_{LIE}) was inversely related to the μ value. This is the first quantitative analysis on the relation between mycelium differentiation and various proteases and protease inhibitors in *Streptomyces* spp. [42, 43].

It was found that the mycelium protein extract of *S. exfoliatus* SMF13 was hydrolyzed effectively by TLP (31,800 Da), which was purified from the culture supernatant of *S. exfoliatus* SMF13. The N-terminal amino acid sequence of TLP was determined to be RVGGTXAAQGNFPFQQXLSM. TLP was inhibited competitively by leupeptin of which the K_i value was 0.015 μM. The data indicated that TLP produced in the stationary phase participated in the degradation of substrate mycelium protein, and the protein hydrolysates were used subsequently for the aerial mycelium growth [44, 45]. The production of TLP was also observed from the stationary growth phase of various species of *Streptomyces* such as *S. aburaviensis*, *S. coelicolor* A3(2), *S. microflavus*, *S. roseus*, *S. lavendulae*, and *S. rochei*.

Leupeptin-inactivating enzyme (LIE) was purified from *S. exfoliatus* SMF13 to 34,700 Da and the N-terminal amino acid sequence of LIE was APTPPDIPLANVPA. LIE hydrolyzed leupeptin (N-acetylleucine-leucine-argininal) to produce N-acetyl-leucine, leucine, and arginal as the products. LIE showed an absolute specificity for peptide bonds with leucine at the P1 position, suggesting that LIE is a leucine-specific protease. LIE activity was found to be a metalloprotease activated by Mg^{2+} or Ca^{2+} ions. Morphological differentiation of *S. exfoliatus* SMF13 was clearly inhibited by the addition LIE inhibitors [46].

From the observations, it was postulated that CTP participated primarily in the proteolysis of the proteinaceous nitrogen source for cell growth. PIs inhibited the activity of TLP; LIE inactivated endogenous leupeptin; TLP functioned as an enzyme involved in the metabolism of cell proteins during morphological differentiation. The cascade roles of the compounds in *S. exfoliatus* SMF13 is assumed to provide a selective advantage in the flexible culture conditions to overcome the limitation of the various external nutrient sources [40–42].

Quantitative Analysis of the Roles of Proteases in the Morphological Differentiation of *Streptomyces albidoflavus* SMF301

As discussed in previous parts, mycelium growth and morphological differentiation of *Streptomyces* spp. are closely related to the availability of protein source in the culture medium. However, studies on the morphological differentiation of *Streptomyces* spp. have been carried out predominantly by solid culture using agar plate media where the concentrations of nutrients are continuously varied with culture time, and also on the location of the culture where the quantitative relationship between nutrient limitation and morphological differentiation has not been well determined by the solid culture.

The species of *Streptomyces* forming spores in submerged culture would provide some advantages on the solid culture for the quantitative analysis of the relation between morphological differentiation and a specific role of a nutrient. Hence, a strain of *Streptomyces* that formed abundant spores in submerged culture was isolated from a soil sample, and the strain was identified as a *Streptomyces albidoflavus* [76]. The characteristics of submerged spores of *S. albidoflavus* SMF301 were compared with those of aerial spores formed on solid culture. The cellular contents of Ca^{2+}, Mg^{2+}, cysteine, and unsaturated fatty acid in submerged spores were significantly higher compared with the aerial spores. On the other hand, aerial spores showed a higher resistance to lysozyme digestion, mild acid treatment, heating, and desiccation. Publications by our group were the first reports on a comparative characterization of spores of *S. albioflavus* formed in submerged and solid cultures [26, 59, 73].

It was also found that *S. albidoflavus* SMF301 produced extracellular proteases under the limitation of amino acids,

and the production of these proteases was closely related to morphological differentiation [48]. The proteases were identified as chymotrypsin-like protease, metalloprotease, and trypsin-like protease. Mycelium growth was linked with the production of CTP, whereas submerged spore formation was accompanied by the production of TLP and MTP. A mutant of *S. albidoflavus* defective in TLP and MTP activity showed the characteristics of a bald phenotype, suggesting that TLP and MTP might participate in the formation of submerged spores. Furthermore, the growth of aerial mycelia and formation of spores were supported by the utilization of substrate mycelia with the involvement of MTP and TLP (Fig. 4).

Mycelial growth and spore formation in submerged culture of *S. albidoflavus* SMF301 were quantitatively analyzed in relation to the proteases formation profiles. Kinetics analysis showed that specific growth rate (μ) was related to the specific rate of CTP production (q_{CTP}). However, specific spore formation rate (q_{spo}) was related to specific rate of TLP production (q_{TLP}). In addition, the formation of submerged spores appeared concurrently with a loss of mycelial production, indicating that TLP played an important role in the endogenous consumption of mycelia [28–30, 74].

The morphological differentiation of *S. albidoflavus* SMF301 in a batch culture was characterized by changes in the pyrolysis mass (PyMs) spectrometry profiles. Curie point PyMs spectra of whole cells were found to vary with growth phase and morphological differentiation. Multivariate statistics and artificial neural network (ANNs) analysis were used to study differentiation of the morphological and physiological states of *S. albidoflavus* SMF301. Furthermore, ANN analysis was applied to the PyMs data to predict the state of differentiation by using two different algorithms; viz., back propagation and a radial basis function classifier. Both the back propagation and the radial basis classifier algorithms succeeded in the identification of the transient state and the degree of differentiation [31, 60].

Molecular Interaction of Proteases and Other Molecules in Relation to Morphological Differentiation of *Streptomyces* spp.

The regulatory mode of the protease expression and the molecules that are interacting with the proteases are the most important topics for elucidation of the role of proteases in relation to the morphological differentiation of *Streptomyces* spp.

It was reported that nucleases were required in the steps for sporulation following after degradation of substrate mycelium DNA and aerial mycelium formation of *S. antibioticus* [21, 62]. A serine protease was involved in the posttranslational modification of a pronuclease to give nuclease activity. Impairment of nuclease processing resulted in failure of aerial mycelium and spore formation in *S. antibioticus* [66]. External feeding of serine protease inhibitors, such as tosyl lysine chloromethyl ketone (TLCK) and leupeptin, resulted in loss of spore formation of *S. exfoliatus*

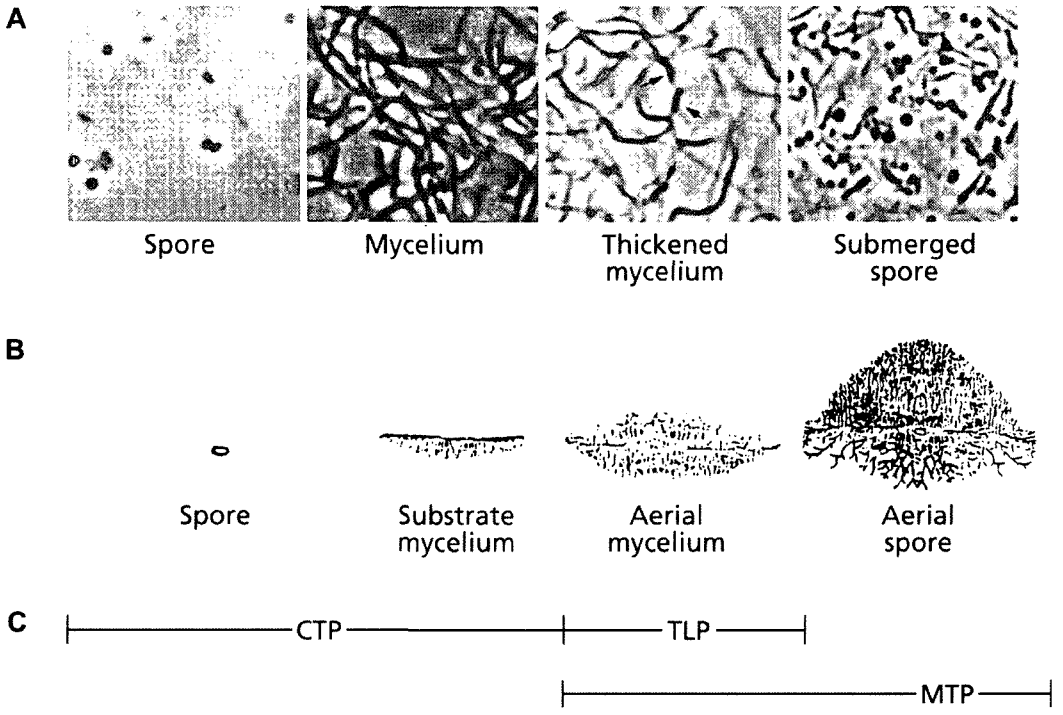

Fig. 4. Morphological differentiation of *S. albidoflavus* SMF301.
A, In submerged; B, solid cultures; C, production phase of CTP, TLP, and MTP.

SMF13 [46] and *S. antibioticus* [66]. The results indicated that a serine protease may be involved in the activation step of nucleases that are essential for DNA processing in the spore formation of *Streptomyces* spp.

It was found that genes encoding trypsin and chymotrypsin (*sprA*, *sprB*, *sprD*, *sprT*, and *sprU*) in *S. griseus* were characterized to be regulated by A-factor-dependent protein (AdpA) [33, 91]. The AdpA has been well recognized to be a key transcriptional activator for the activation of a number of genes required for secondary metabolism and morphological differentiation in *Streptomyces* spp [20, 67]. Mutants deleted in the double genes encoding trypsin (*ΔsprT*, *ΔsprU*) and triple genes for chymotrypsin (*ΔsprA*, *ΔsprB*, *ΔsprD*) showed normal growth but did not give any specific morphological defects [33, 91]. However, another AdpA regulon protease, reported as *Streptomyces griseus* metalloprotease A (SgmA), seems to be involved in morphological differentiation. Disruption of a gene (*sgmA*) encoding SgmA resulted in some what of a delay of the aerial mycelium, indicating that SgmA was involved in morphological differentiation by the degradation of proteins in substrate hyphae for reuse in aerial hypha formation [32]. The data confirm the previous results that were obtained from *S. exfoliatus* SMF13 and *S. albidoflavus* SMF301.

Since completion of the study of the genomics of *S. coelicolor* [10], genes for proteases and protease inhibitors were analyzed in conjunction with the proteomics of the membrane-associated proteins and secreted proteins [36, 37]. From this study, the regulation mechanism of the protease inhibitor (SSI family, *Streptomyces* trypsin inhibitor) was analyzed. In *S. coelicolor*, a protease inhibitor (STI) was considered to be encoded by the SCO0762 but not by SCO4010, which was considered to be a pseudogene. Its expression and the protease inhibition activity were very much lower compared with other protease inhibitors of SSI [34]. STI has a highly conserved amino acids sequence with other SSIs and is identical to the SLP1 that was identified from *S. lividans* [93]. From a proteomics approach, STI was found to be completely absent from the *bldA* mutant. This dependence was shown to be mediated *via* the TTA-containing regulatory gene *adpA*, a developmental gene that is responsible for the effects of *bldA* on differentiation. Furthermore, SSI regulation by *bldA*-AdpA seems to be conserved in some *Streptomyces* (*S. albogriseolus* S-3253 and *S. venezuelae*). The AdpA-binding consensus upstream of the SSI coding gene was conserved in these strains. In addition, mutation of the SCO0762 gene resulted in abolishment of detectable trypsin-protease inhibitory activity. STI is a sole inhibitor protein that represents inhibition

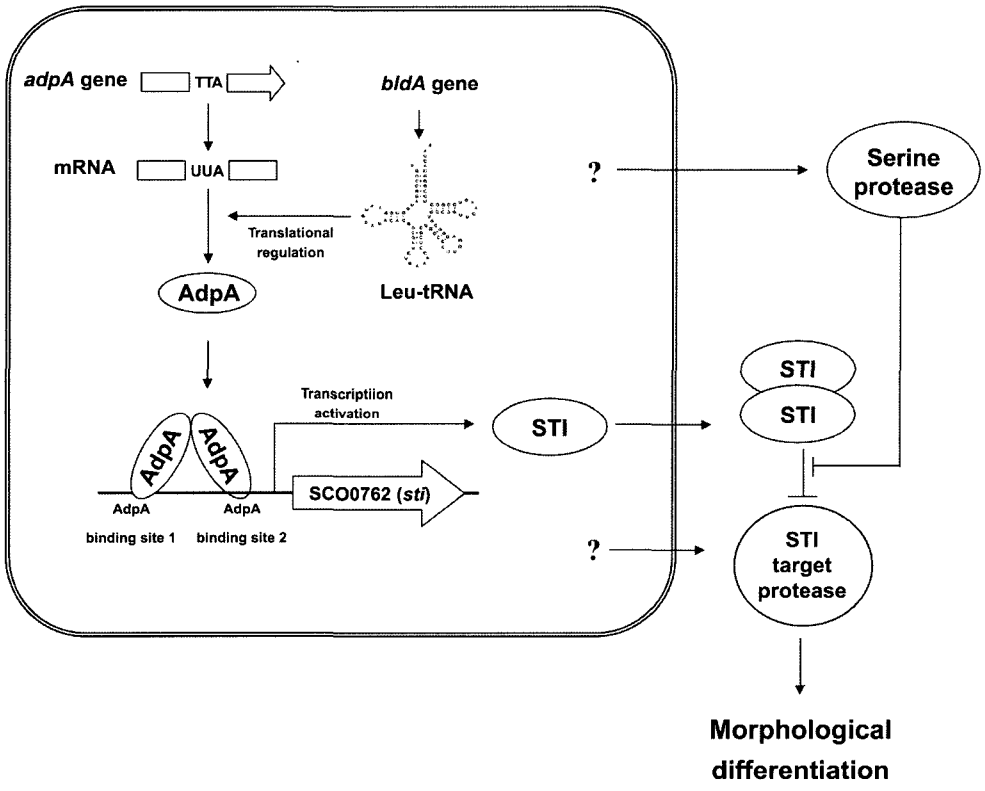

Fig. 5. Proposed model of differentiation regulation by proteases and protease inhibitor (STI) in *Streptomyces coelicolor*.
This model is based on protease and its inhibitor studies of *S. griseus*, *S. antibioticus*, and *S. exfolaitus* SMF13.

activity in the extracellular region. The biological role of STI is still not clear, because disruption of SCO0762 did not result in any obvious morphological defect [36].

The biological function of PI including STI is assumed as a key regulator in the extracellular region in two ways. First, PI controls extracellular protease activity for proper growth of subtrate mycelium [34]. Second, PI may inhibit specific proteases involved in translational modification of essential protein for maintaining an accurate cell cycle (Fig. 5). Since some serine proteases were found to be involved in the process of morphological differentiation of *Streptomyces* spp., the interaction mode between the serine proteases and protease inhibitor is an important aspect to be discovered.

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